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BRIEF REPORT

Characterization of a flowering cherry strain of *Cherry necrotic rusty mottle virus*

R. Li · R. Mock

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Abstract The host range and complete nucleotide sequences of two *Cherry necrotic rusty mottle virus* (CNRMV) isolates (FC4 and FC5) infecting flowering cherry accessions imported from Japan are described. Of the plants tested, cherry, peach, apricot and almond became infected, but only sweet cherry cv. 'Canindex', Nanking cherry and apricot cv. 'Tilton' showed a mild foliar mottle. The genomic sequences of CNRMV-FC4 and CNRMV-FC5 are 8,430 and 8,429 nt in length, excluding the 3′ poly (A) tail. They contain seven open reading frames encoding for a putative virus replicase, "triple gene block" proteins, a coat protein and two proteins with unknown functions. The two CNRMV-FC isolates share 96% identity in the genomic sequences, and their genome organizations are virtually identical to that of a German CNRMV isolate (CNRMV-GER). However, they differ from CNRMV-GER by 14% in the overall nucleotide sequence and 2% (ORF2) to 30% (ORF5a) in the derived amino acid sequences of individual gene products.

Cherry necrotic rusty mottle virus (CNRMV) and the closely-related *Cherry green ring mottle virus* (CGRMV) are unassigned members in the family *Flexiviridae*, a large and diverse group of flexuous, filamentous, single-strand RNA viruses infecting plants [1]. The family is comprised of the genera *Allexivirus*, *Capillovirus*, *Carlavirus*, *Foveavirus*, *Mandarivirus*, *Potexvirus*, *Trichovirus*, *Vitivirus* and six unassigned members including CNRMV. The main characteristics of members of the family are a common alpha-like replicase containing conserved methyltransferase (MTR), helicase (HEL) and RNA-dependent RNA polymerase (RdRp) motifs, one or more movement proteins (MP), and a single coat protein (CP). The genomes of CNRMV and CGRMV are approximately 8.4 kb and contain seven open reading frames (ORFs), which differ from those of *Foveavirus* and *Potexvirus* in having two nested putative ORFs (ORF2a and 5a) in their genomes [1, 3, 16, 21].

CNRMV infects sweet cherry (*Prunus avium*) and has been reported in North America, Europe and Japan [3, 7, 13, 16, 19]. Diseased plants show brown angular necrotic spots, rusty chlorotic areas, shot holes of the leaves, and blisters, gum pockets and general necrosis of the bark [19]. The virus is transmitted by grafting but not mechanically through plant sap. The CNRMV-FC isolates were first detected in five of six symptomless flowering cherry accessions (*P. serrulata* and hybrids) imported from Japan in 2003, using a RT-PCR assay [10]. Analysis of the CP sequences obtained from the RT-PCR products revealed that these isolates were very similar to a sweet cherry isolate of CNRMV from German (CNRMV-GER) [16]. However, they did not induce any obvious symptoms on sweet cherry cv. 'Sam', a woody indicator used to detect CNRMV. Furthermore, a pair of degenerate primers based on the conserved regions of the 5′-portions of available CNRMV and CGRMV sequences did not amplify any products from these isolates, indicating they were different from CNRMV-GER. Two isolates, FC4 and FC5, also caused different host reactions on the inoculated flowering cherry cv. 'Kwanzan' and peach (*P. persica*) cv. 'GF305'. Here, we report the host reactions of these two CNRMV-FC isolates in several *Prunus* spp., their complete nucleotide sequences and their phylogenetic relationships with other viruses in the family *Flexiviridae*.

R. Li (✉) · R. Mock
USDA-ARS, National Germplasm Resources Laboratory,
Beltsville, MD 20705, USA
e-mail: Ruhui.Li@ars.usda.gov

Table 1 Comparison of host reactions of the CNRMV-FC isolates and CNRMV-WP192

Plant species	FC4		FC5		WP192	
	Bioassay	RT-PCR[a]	Bioassay	RT-PCR	Bioassay	RT-PCR
Prunus avium cv. 'Bing'	NS	+	ND	ND	Mo	+
P. avium cv. 'Sam'	NS	+	NS	ND	Mo	+
P. avium cv. 'Canindex'	MMo	+	ND	ND	SMo	+
P. armeniaca cv. 'Tilton'	MMo	+	Y, LD	+	NS	+
P. cerasifera	NS	−	NS	−	NS	−
P. dulcis cv. 'Peerless'	NS	+	ND	ND	NS	−
P. mandshurica	NS	−	NS	−	NS	−
P. mahaleb	NS	+	NS	−	NS	−
P. persica cv. 'Boone County'	NS	+	ND	ND	NS	+
P. persica cv. 'Lovell'	ND	ND	NS	+	ND	ND
P. persica cv. 'GF305'	NS	+	CV/-?	+	NS	+
P. salicina cv.'Shiro'	NS	−	NS	−	NS	−
P. serrulata cv. 'Kwanzan'	NS	+	NV, ME/-?	+	NS	+
P. tomentosa	MMo	+	NV, E, Mo	+	Mo	+

NS no symptoms, *Mo* mottle, *MMo* mild mottle, *Smo* severe mottle, *Y* yellowing, *LD* leaf distortion, *CV* chlorotic vein, *NV* necrotic vein, *-?* = no symptoms in one test, *ME* mild epinasty, *E* epinasty, *ND* no data

[a] RT-PCR [10] was used to detect viral infection

To determine their experimental host ranges, FC4 and FC5 were graft-inoculated onto three trees of thirteen and ten Prunus woody indicator plants, respectively (Table 1). A sweet cherry isolate, WP192, was included as a positive control. After inoculation, plants were kept for symptom observation in an insect-proof greenhouse for two years. Total nucleic acids were extracted from each inoculated plant and tested by RT-PCR to confirm the virus transmission. The results indicated that these isolates infected most inoculated plants, but with slightly different host range and reactions (Fig. 1; Table 1). FC4 caused a symptomless infection of Mahaleb cherry (*P. mahaleb*), but WP192 and FC5 did not. FC4 infected almond (*P. dulcis*), but WP192 did not. All three isolates induced mottle symptoms on Nanking cherry (*P. tomentosa*) of varying intensities. FC4 induced mottle symptom on sweet cherry cv. 'Canindex', similar to, but milder than that caused by WP192, but it did not induce any symptoms on cvs. 'Bing' and 'Sam'. Unlike WP192, both FC4 and FC5 caused symptomatic infection on apricot (*P. armeniaca*), but the symptoms were different. Biological and serological assays later showed that the source plant of FC5, but not FC4, was also infected by *American plum line pattern virus*, which probably accounted for the differences in the host reactions. None of the three CNRMV isolates infected Manchurian apricot (*P. mandshurica*) or plums (*P. cerasifera* and *P. salicina*). Three CNRMV isolates and one CGRMV isolate have produced variable results from year to year when inoculated to Kwanzan and GF305, indicating that symptom induction by these viruses on the indicators is influenced by environmental conditions. Therefore, application of RT-PCR is important for detection of these viruses in virus indexing programs.

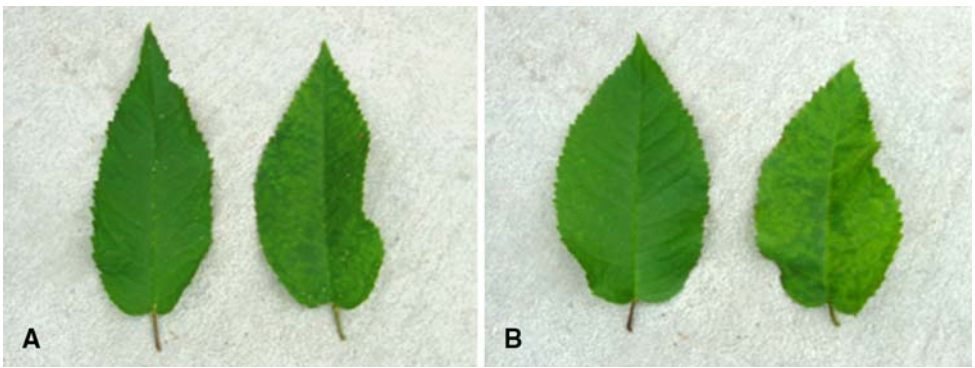

Fig. 1 Comparison of the host reactions caused by CNRMV-FC4 (left leaves) and CNRMV-WP192 (*right* leaves) on the inoculated woody indicators, sweet cherry cv. 'Bing' (**a**) and 'Sam' (**b**). CNRMV-FC4 did not induce any visible symptoms, whereas CNRMV-WP192 caused foliar mottle on the plants 2 months after graft inoculation

Total RNA was extracted from tissues of the infected plants according to the CTAB method described by Li et al. [11]. Three types of primers were used to obtain the RT-PCR products for cloning of CNRMV-FC isolates: (1) degenerate or consensus primers designed to anneal to conserved regions of the CNRMV and CGRMV sequences available in the GenBank database, (2) specific GER primers designed according to the CNRMV-GER sequence, and (3) specific FC5 primers designed according to CNRMV-FC5 sequences obtained during this study. RT-PCR was carried out using the OneStep RT-PCR kit (QIAGEN Inc., Valencia, CA, USA). The nucleotide sequences corresponding to the 5′- and 3′-terminal regions were obtained using the FirstChoice® RLM-RACE kit (Ambion Inc., Austin, TX, USA). The amplified PCR products were cloned into the pGEM®-T Easy Vector (Promega, Madison, WI, USA). Plasmid DNA from 2–6 clones of each ligation were isolated using the Fast-Plamid® Mini kit (Eppendorf, Westbury, NY, USA) and sequenced on both strands ((MacrogenUSA, Rockville, MD, USA).

The sequences obtained were assembled by CAP3 Sequence Assembly Program at the PBIL sever (http://pbil.univ-lyon1.fr/cap3.php) [6]. Database searches were performed using Fast3 at the EMBL-EBI (http://www.ebi.ac.uk/fasta33/) [15], and pairwise alignments of sequences were performed using the EMBOSS Pairwise Alignment Algorithms at the EMBL-EBI (http://www.ebi.ac.uk/emboss/align/) [4]. Multiple alignments of the CP amino acid sequences were performed by the neighbor-joining algorithm as implemented in CLUSTAL_X [17], and the resulting alignments were analyzed using MEGA version 3.1 [9]. Phylogenetic trees were constructed using the neighbor-joining algorithm with 100 bootstrap replicates. The viruses used in sequence alignments and phylogenetic analysis were as follows: *Apple chlorotic leaf spot virus* (ACLSV, M58152), *Apple stem grooving virus* (ASGV, D14995), *Apple stem pitting virus* (ASPV, D21829), *Banana mild mosaic virus* (BanMMV, AF314662), *Blueberry scorch virus* (BlScV, NC_003499), *Cherry green ring mottle virus* (AF017780, AJ291761), *Cherry mottle leaf virus* (CMLV, AF170028), *Cherry necrotic rusty mottle virus* (CNRMV-GER, AF237816), *Cherry virus A* (CVA, X82547), *Citrus leaf blotch virus* (CLBV, AJ318061), *Clover yellow mosaic virus* (ClYMV, D29630), *Garlic virus A* (GarV-A, AB010300), *Grapevine virus A* (GVA, X75433), *Grapevine virus B* (GVB, X75433), *Indian citrus ring spot virus* (ICRSV, AF406744), *Lily symptomless virus* (LSV, NC_005138), *Potato virus* X (PVX, NC_001455), *Rupestris stem pitting-associated virus* (RSPaV, AF026278), *Shallot virus X* (ShVX, NC_003795) and *Sugarcane striate mosaic-associated virus* (SCSMaV, AF315308) (Fig. 2).

The complete genomic sequences of CNRMV-FC4 and FC5 are 8,430 and 8,429 nucleotides (nt), respectively, excluding the poly (A) tail at the 3′-terminus (GenBank accession no. EU188438 and EU188439). They contain seven ORFs, which are organized in the same arrangement as those of known CNRMV and CGRMV isolates [3, 16, 21]. Sequence comparisons of the genomes, non-translated regions (NTRs) and all putative gene products between FC4 and other CNRMV isolates, representative members of all genera and unassigned viruses in the family *Flexiviridae* were calculated (Table 2). Except for the ORF4, the sequence identities between two FC isolates on the genomic sequence, the NTRs and other ORFs are higher than those between isolates FC4 and GER, reflecting their host and geographical origins. However, two FC isolates still retain high homologies to the GER isolate, indicating that the three isolates belong to the same species [1]. The sequence identities between CNRMV-FC4 and two CGRMV isolates are much higher than those between CNRMV-FC4 and other viruses, indicating that these two viruses are also closely related (Table 2).

The 5′-NTRs of all three CNRMV isolates are 117 nt. The sequence 5′-GAAAA-3′, a pentamer nucleotide sequence found in the 5′-end of CGRMV and most potexviruses [18, 20, 21], is located at nt 3–7 in their genomes. The 3′-NTRs of FC4 and FC5 are 189 and 188 nt, respectively, shorter than that of CNRMV-GER (191 nt). The putative polyadenylation signal, AAUAAA, is located at positions 8,352–8,357 nt of the FC isolates. The hexanucleotide motif "ACUUAA" found in the 3′-NTR of the North American isolate of CGRMV (CGRMV-NA) is not found in the CNRMV isolates [21].

ORF1 (118–6,234 nts) of the FC isolates encodes a putative replicase of 2,038 amino acids (aa) with a calculated Mr of 231 kDa., very similar to that of CNRMV-GER. Multiple alignment of amino acid sequences revealed that the CNRMV replicase shares many common features with those of CGRMV, foveaviruses and carlaviruses. The CNRMV replicase contains motifs for type I MTR (aa 70–270), a variable region (aa 645–700), ovarian tumor (OTU)-like cysteine protease (OTU, aa 931–1047), carlavirus endopeptidase (Peptidase_C23, aa 1050–1138), HEL (aa 1226–1486) and RdRp_2 (aa 1604–2019) [2, 5, 8, 12, 18]. The OTU homologue has been found in eukaryotes, fungi, bacteria, a few animal viruses and some plant viruses including members of the genera *Foveavirus* and *Carlavirus* and three of six unassigned viruses in the family *Flexiviridae*, and its function is unknown [2, 12]. Another conserved domain found in the CNRMV replicase is a AlkB homologue (aa 783–884) of the 2-oxoglutarate- and Fe(II)-dependent oxygenase superfamily. The AlkB-like genes are widespread in eukaryotes and bacteria and have also been identified in plant viruses including many viruses

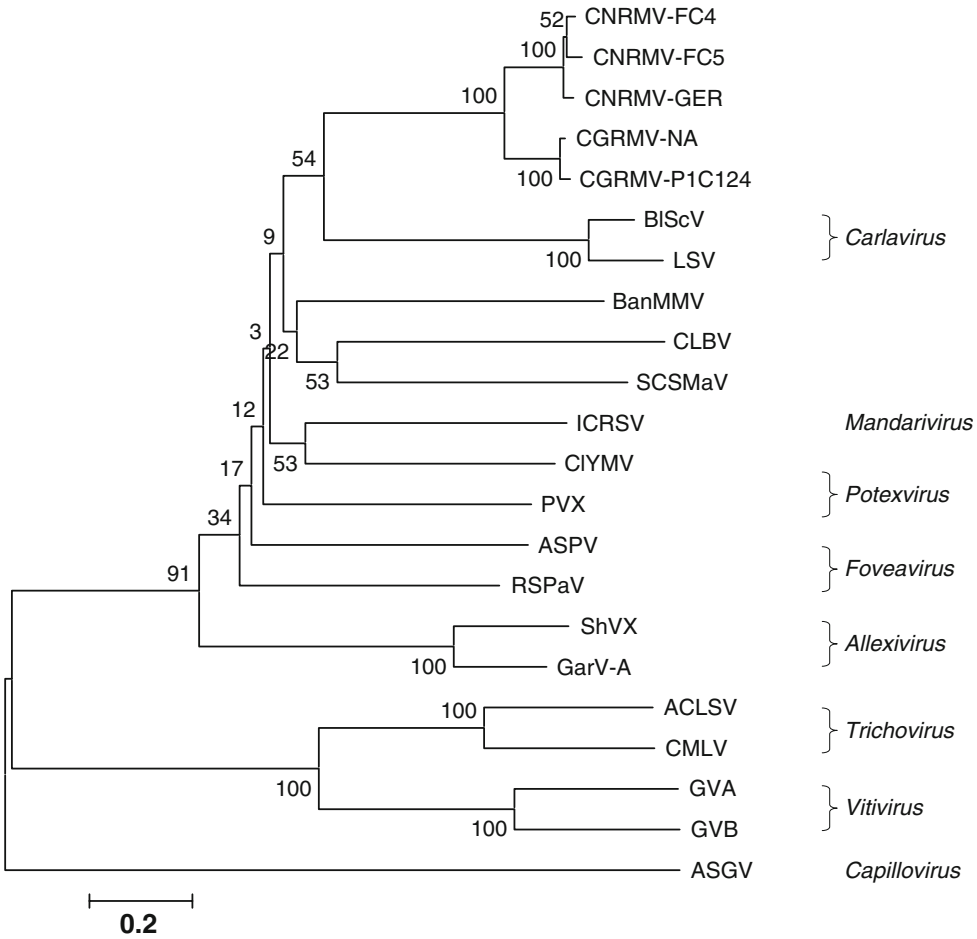

Fig. 2 Unrooted phylogenetic trees derived from aligned CP amino acid sequences of three *Cherry necrotic rusty mottle virus* (CNRMV) isolates, two *Cherry green ring mottle virus* (CGRMV) isolates, selected viruses of the different genera and other unassigned members in the family *Flexiviridae* (see text for details). The alignments were generated using ClustalX, and the trees were constructed using MEGA 3.1

Table 2 Comparison of nucleotide (nt) and amino acid (aa) sequence identity (%) between a *Cherry necrotic rusty mottle virus*-flowering cherry isolate 4 (CNRMV-FC4) and other CNRMV isolates, *Cherry green ring mottle virus* (CGRMV) isolates and representative members of the family *Flexiviridae*

Virus	Genomic sequence (nt)	5′-NTR (nt)	ORF1 (aa)	ORF2 (aa)	ORF3 (aa)	ORF4 (aa)	ORF5 (aa)	ORF2a (aa)	ORF5a (aa)	3′-NTR (nt)
CNRMV-FC5	95	97	96	99	96	94	97	94	89	95
CNRMV-GER	86	96	91	98	93	96	96	91	70	93
CGRMV-NA	67	81	70	77	56	71	75	49	38	76
CGRMV-P1A	68	81	69	77	58	68	74	49	36	75
ACLSV	48	42	29	–	–	–	7	–	–	43
ASGV	45	19	21	–	–	–	18	–	–	42
ASPV	51	21	35	45	26	31	17	–	–	36
BlScV	50	32	34	34	33	35	26	–	–	22
GVA	47	43	27	–	–	–	12	–	–	20
ICRSV	44	38	22	28	35	26	26	–	–	13
PVX	47	48	19	31	37	24	22	–	–	27
ShVX	46	46	20	29	34	8	23	–	–	31
BanMMV	50	40	31	34	41	33	27	–	–	27
CLBV	49	42	28	–	–	–	17	–	–	47
SCSMaV	51	37	31	25	26	30	24	–	–	44

No corresponding ORF is present

in the family *Flexiviridae* and ampeloviruses in the family *Closteroviridae* [2].

ORF2 (nt 6234–6902), ORF3 (nt 6903–7250) and ORF4 (nt 7180–7383) form the 'potex-like' cell-to-cell movement-associated 'triple gene block' (TGB) found in CGRMV, BanMMV, SCSMaV and in the genera *Allexivirus*, *Carlavirus*, *Foveavirus*, *Mandarivirus* and *Potexvirus* [14, 18]. These ORFs encode three polypeptides of 222, 115 and 67 aa with calculated Mr of 25, 12 and 7 kDa, respectively. The TGBp1 is a viral RNA helicase (HEL) of the superfamily I, with a NTP-binding domain (aa 26–36) at the N-terminus, and is highly conserved among CNRMV isolates as well as between CNRMV and CGRMV (Table 2). The TGBp2 and TGBp3 are transmembrane proteins with conserved central domains (14).

ORF5 (nt 7438–8241) encodes a putative CP of 267 aa with a calculated Mr of 30 kDa. The protein is also highly conserved among CNRMV isolates as well as between CNRMV and CGRMV (Table 2) and contains the CP signature of potexviruses and carlaviruses (aa 190–205) [3, 16, 20].

ORF2a (nt 6262–6621) and ORF5a (nt 7460–7933), two nested ORFs conserved in both CGRMV and CNRMV [3, 16, 21], potentially encode polypeptides of 119 and 157 aa with calculated Mr of 13 and 18 kDa, respectively. The ORF2a-encoded polypepetide is very rich in leucine residues and has a distant similarity to the ATPase subunit 6 of some fungal mitochondria and bacteria. No sequence similarity was found between the ORF5a-encoded protein and other known proteins in GenBank, and it is the least conserved among the CNRMV isolates and between CNRMV and CGRMV (Table 2). The presence of these unusual ORFs is the hallmark of CNRMV and CGRMV, although the existence of these proteins is a matter of speculation and their potential roles are not known [1]. The preservation of these ORFs suggests that the proteins are likely to be essential and possibly related to some unique biological features of the CNRMV and CGRMV.

Phylogenetic analysis based on CP sequences was conducted to examine the relationship of the CNRMV isolates, representative members of different genera and unassigned viruses in the family *Flexiviridae* (Fig. 2). The FC isolates grouped tightly with CNRMV-GER, and together they formed a distinct cluster at species level. CNRMV was most closely related to CGRMV, and the two viruses formed a distinct cluster at genus level. CNRMV and CGRMV were more closely related to viruses in the genus *Carlavirus* than those in the genera *Capillovirus*, *Trichovirus* and *Vitivirus* (Fig. 2).

In summary, the sequence analyses of the CNRMV-FC isolates clearly indicate that they belong to the species *Cherry necrotic rusty mottle virus*. However, they differ from sweet cherry isolates GER and WP192 in having different natural and experimental hosts and causing different symptoms on several woody indicator plants [16]. Therefore, the CNRMV-FC isolates together should be considered a flowering cherry strain, separating from the sweet cherry strain typified by CNRMV-GER. CNRMV is closely related to CGRMV, and the two viruses share several features with, but are also distinct from, other viruses in the family *Flexiviridae*. These common and unique features enhance our understanding of flexiviruses and will be useful in taxonomic assignment of these viruses.

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